Evaluation of methods for cultivating limbal mesenchymal stromal cells

Running Title: Cultivation of limbal mesenchymal stromal cells

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Abstract

Background: Mesenchymal stromal cells (MSC) with similar properties to bone marrow derived mesenchymal stromal cells (BM-MSC) have recently been grown from the limbus of the human cornea. We presently contribute to this novel area of research by evaluating methods for culturing human limbal MSC (L-MSC). *Methods:* Four basic strategies are compared: serum-supplemented medium (10% foetal bovine serum; FBS), standard serum-free medium supplemented with B-27, epidermal growth factor, and fibroblast growth factor 2, or one of two commercial serum-free media including Defined Keratinocyte Serum Free Medium (Invitrogen), and MesenCult-XF® (Stem Cell Technologies). The phenotype of resulting cultures was examined using photography, flow cytometry (for CD34, CD45, CD73, CD90, CD105, CD141, CD271), immunocytochemistry (α-sma), differentiation assays (osteogenesis, adipogenesis, chrondrogenesis), and co-culture experiments with human limbal epithelial (HLE) cells. *Results:* While all techniques supported to varying degrees establishment of cultures, sustained growth and serial propagation was only achieved in 10% FBS medium or MesenCult-XF® medium. Cultures established in 10% FBS medium were 70-80% $CD34^-/CD45^-/CD90^+/CD73^+/CD105^+$, approximately 25% $\alpha\text{-sma}^+$, and displayed multi-potency. Cultures established in MesenCult-XF® were >95% $CD34^-/CD45^-/CD90^+/CD73^+/CD105^+$, 40% $CD141^+$, rarely expressed α-sma, and displayed multi-potency. L-MSC supported growth of HLE cells, with the largest epithelial islands being observed in the presence of MesenCult-XF®-grown L-MSC. All HLE cultures supported by L-MSC widely expressed the progenitor cell marker ΔNp63, along with the corneal differentiation marker cytokeratin 3. *Conclusions:* We conclude that MesenCult-XF® is a superior culture system for L-MSC, but further studies are required to explore the significance of CD141 expression in these cells.

Keywords

Cornea; Epithelium; Keratocytes; Limbus; Mesenchymal Stromal Cells

Abbreviations

APC - Allophycocyanin

α-sma – Alpha smooth muscle actin

BM-MSC – Bone marrow mesenchymal stromal cells

CFE – Colony forming efficiency

CK3 – Cytokeratin 3

DK-SFM – Defined Keratinocyte Serum Free Medium

DMEM – Dulbecco's Modified Eagle's Medium

EGF – Epidermal growth factor

FBS – Foetal bovine serum

FGF-2 – Fibroblast growth factor 2

FITC – Fluorescein isothiocyanate

HLE – Human limbal epithelial

HLS – Human limbal stromal

L-MSC – Limbal mesenchymal stromal cells

LSCD – Limbal stem cell deficiency

MSC – Mesenchymal stromal cell

NGS – Normal goat serum

PBS – Phosphate buffered saline

PE - Phycoerythrin

Introduction

The limbus, or edge, of the cornea contains stem cells for regenerating the corneal epithelium (1). Diseases and injuries affecting the limbus can lead to a condition known as limbal stem cell deficiency (LSCD). The primary symptom of LSCD is loss of the corneal epithelium, which subsequently leads to chronic inflammation and scarring of the ocular surface. Advances in the treatment of LSCD have been achieved through use of cultured epithelial grafts prepared from limbal tissue. Traditionally, these epithelial grafts are grown from limbal tissue explants cultured on donor amniotic membrane (2-4). Alternatively, dissociated limbal tissue has been subjected to *ex vivo* expansion in the presence of growth-arrested murine fibroblast feeder cells (5-7). Logically, the therapeutic benefits of cultured limbal grafts are primarily due to the presence of a robust population of poorly differentiated epithelial cells, but these are not the only cell type present.

Cultures of limbal epithelium contain variable numbers of contaminating human limbal stromal (HLS) cells (8). Based upon simple morphological criteria, these contaminating stromal cells have been referred to as “fibroblasts” or “keratocytes”, but a recent comparison with bone marrow derived mesenchymal stem/stromal cells (BM-MSC) has revealed a similar profile of cell surface markers and evidence of multi-potency (8). The discovery of limbal mesenchymal stromal cells (L-MSC) raises significant questions regarding the potential role of stromal cells in cultured limbal grafts and may well lead to improved strategies for corneal tissue engineering. To this end, we have presently evaluated a range of different culture methods with the view to optimising L-MSC yield and purity for subsequent clinical use.

Current techniques for growing L-MSC are based upon use of serum-supplemented growth medium (8-10). While providing an effective source of cell attachment and growth factors, the properties of serum can vary significantly from batch to batch or between donors

thus leading to inconsistent results. Moreover, serum contains factors that promote cell differentiation, which could potentially lead to a reduction in product efficacy. Thus an ideal method for growing L-MSC would be one that supports high yields with minimal differentiation. A review of methods for growing cells (MSC or keratocytes) from either the limbal or corneal stroma revealed a variety of strategies for exploration (Table 1). To begin, cultures that are simply maintained in serum-supplemented medium display characteristics similar to those of BM-MSC; however, they also contain differentiated cells including myofibroblasts (8-10). Secondly, cultures can be initiated in the presence of serum then switched to serum-free medium containing a number of defined growth factors (11). Alternatively, completely serum-free techniques can be used (12-14). Use of serum-free media with corneal stromal cells appears to have maintained these cells in a poorly differentiated state, but the degree of proliferation observed in these cultures is not clear.

Based upon consideration of the above, we have evaluated four basic strategies for growing L-MSC: serum supplemented medium, conventional serum-free medium supplemented with defined growth factors previously used to support stromal cells, and two commercial serum-free media, Defined Keratinocyte Serum-Free Medium (DK-SFM; Invitrogen) and MesenCult-XF® (Stem Cell Technologies). Our results demonstrate marked differences in L-MSC phenotype under each growth condition and provide evidence of a novel marker for L-MSC.

Materials and methods

Establishment of cultures from limbal stroma

Human corneoscleral rims were obtained with ethics approval and donor consent from the Queensland Eye Bank (QEB), Brisbane, Australia. Prior to digestion, the tissue was dissected

down to a 1.5 - 2 mm diameter across the limbal transition between transparent cornea and white sclera. The tissue was then washed in three changes of phosphate buffered saline (PBS) to remove storage medium, then digested with 0.25% dispase (Invitrogen, VIC, Australia) for 1 h at 37 ºC to assist removal of HLE cells. In later experiments the HLE was retained for co-culture experiments with stromal cells (see below). The remaining stroma was digested in 1 mg/ml Collagenase Type I (Invitrogen, VIC, Australia) for 48 h in DMEM/F12 (Invitrogen, VIC, Australia). The dissociated stromal cells were washed and initially seeded at a density of 5×10^3 cells/mL where possible under four different culture conditions. The first culture condition consisted of DMEM/F12 medium supplemented with 10% foetal bovine serum (FBS; Hyclone Thermo Scientific, VIC, Australia), thus mimicking the approach most commonly used in previous studies including our own (8-10). For simplicity this first medium is subsequently referred to as 10% FBS medium. The second approach (hereafter referred to as 2% B-27 medium) was based upon the method of Mimura et al. (14), in which cultures are grown in DMEM/F12 medium supplemented with 2% B-27, 1% insulin transferrin selenium (ITS) supplement, 20 ng/mL epidermal growth factor (EGF) and 40 ng/mL fibroblast growth factor 2 (FGF-2) (all Invitrogen, VIC, Australia). The third technique was based upon the method of Kawakita et al. (13) using Defined Keratinocyte Serum-Free Medium (DK-SFM; Invitrogen, VIC, Australia), and was compared to MesenCult®-XF culture system (Stem Cell Technologies, VIC, Australia), a serum-free culture system designed for cultivation of human-derived MSC. All media were additionally supplemented with 1% penicillin/streptomycin solution (Invitrogen, VIC, Australia). Finally, subsequent studies investigated the effects of establishing cultures in 10% FBS medium, then subculturing in each of the original serum-free media as well as DMEM/12 medium supplemented with 10 ng/mL FGF-2 alone.

Establishment of primary BM-MSC cultures

Human BM-MSC were used as a positive control for studies involving assessment of MSC phenotype and differentiation potential with human L-MSC. All BM-MSC experiments were approved by the Mater Health Services Human Research Ethics Committee. Briefly, up to 20ml of bone marrow aspirate was removed from the iliac crest from a healthy donor. The sample was then underlayed with Ficoll and centrifuged at 535 g for 20 min at 20°C, with no brake. The interface layer was then removed and transferred to a separate tube. This was then washed with PBS and centrifuged at 350 g for 5 min with brake. The supernatant was then discarded and the cell pellet resuspended and plated in culture with DMEM (low glucose; Invitrogen, VIC, Australia) supplemented with 20% FBS and 1% penicillin/streptomycin/glutamine solution (Invitrogen, VIC, Australia).

Flow cytometry

Cellular phenotype analysis was performed on L-MSC and BM-MSC by flow cytometry using fluorescently labelled mouse anti-human antibodies as follows: IgG1 κ isotype control phycoerythrin (PE) (MOPC-21), CD34 fluorescein isothiocyanate (FITC) (581/CD34, IgG1 κ), CD45 PE-cyanine dye (Cy7) (HI30, IgG1 κ), CD73 allophycocyanin (APC) (AD2, IgG1 κ), CD90 APC (5E10, IgG1 κ), CD105 PE (SN6, IgG1 κ), and CD141 PE (1A4, IgG1 κ). All antibodies were purchased from Becton-Dickinson (BD Pharmingen, CA, USA) or Ebioscience (in the case of CD73 and CD105; Jomar Bioscience, SA, Australia). Viability was assessed by 7-amino-actinomycin D incorporation (BD Viaprobe cell viability solution). Antibody concentrations were used according to manufacturers' instructions. Fifty thousand events were collected on a BD LSRII (BD Biosciences, CA, USA) and analysed using FlowJo software (Tree Star Inc., OR, USA). Cell subsets were defined as follows: MSC ($CD45^-/CD90^+/CD73^+/CD105^+$) (15,16), putative keratocyte marker ($CD34^+$) (17,18),

fibroblasts ($CD90^+$), myofibroblasts ($CD90^+/\alpha\text{-}sma^+$), putative epithelial cell marker ($CD141^+$) (19,20), vascular endothelial cells ($CD34^+/CD141^+$), bone marrow derived cells ($CD45^+$) and glial cells ($CD271^+$).

MSC differentiation studies

Evidence of multi-potency was investigated by growing cultures of HLS cells (initially cultivated in 10% FBS and MesenCult-XF®) under osteogenic, chondrogenic, and adipogenic inductive conditions. Cultures of human BM-MSC were used as a positive control. For osteogenic conditions, cells were seeded at 10^4 cells/cm^2 and grown for 3 weeks in osteogenic medium (DMEM medium supplemented with 0.1 μM dexamethasone, 10 mM β-glycerol phosphate, 200 μM L-ascorbate-2-phosphate, 4 mM calcium chloride $CaCl_2$, 10% FBS and 1% penicillin/streptomycin/glutamine (PSG) solution) before fixation in 4% buffered formalin and staining with Alizarin red. For adipocyte differentiation, cells were initially seeded at 5×10^4 per well (24 well plate) in 10% FBS media and cultured overnight before being grown for 3 weeks in adipocyte differentiation medium (DMEM medium supplemented with 0.5 mM IMBX, 60 μM indomethacin, 5 μg/mL insulin, 1 μM dexamethasone, 10% FBS, and 1% PSG), fixed in formalin, and stained for lipid formation using Oil-red O. For chondrocyte differentiation, 5×10^5 cells were collected and placed in a 15 mL tube, centrifuged at 300 g for 5 min to form a cell pellet, and the overlaying supernatant gently replaced with 500 μl of chondrocyte differentiation media (DMEM medium supplemented with 0.1 μM dexamethasone, 0.2 mM ascorbic acid 2 phosphate, 1 mM sodium pyruvate, 40 ug/mL proline, 10 ng/mL TGF-β3, 5 μg/mL ITS, and 1% PSG solution). After 3 weeks culture, pellets were fixed in formalin and processed for either paraffin or frozen sectioning before staining with Alcian Blue and performing immunohistochemistry for Collagen II.

Immunostaining

Immunocytochemistry was performed to determine the expression of α-sma in passage 2 HLS cell cultures grown in 10% FBS and MesenCult-XF®, and later to examine the phenotype of HLE cells in co-culture experiments (cytokeratin 3 and ΔNp63). Briefly, cultures were fixed in 10% buffered formalin, briefly washed in PBS, then blocked and permeabilised in 0.1% Triton X-100 with 5% normal goat serum (NGS) in PBS for 1 h at 37 ºC. Cultures were then incubated separately with each primary antibody for 1 hr in 1% NGS/PBS. An Alexa Fluor 488 goat anti-mouse IgG secondary antibody (Invitrogen, VIC, Australia) was used to detect antibodies to α-sma (Dako Australia Pty Ltd, VIC, Australia) and cytokeratin 3 (CK3; Millipore, VIC, Australia), and an Alexa Fluor 594 goat anti-rabbit secondary antibody (Invitrogen, VIC, Australia) was applied to detect antibody to ΔNp63 (Biolegend, Australian Biosearch, WA, Australia). All secondary antibodies were applied for 1 h at 37 ºC and all cultures were counterstained with Hoechst nuclear dye for 15 minutes.

Immunohistochemistry was performed on L-MSC and BM-MSC chondrogenic differentiation pellets for Collagen Type II (II-II6B3; Developmental Studies Hybridoma Bank, Iowa, USA) with a VECTASTAIN Elite ABC-Peroxidase kit (Vector Laboratories, Abacus ALS, QLD, Australia) according to manufacturers' instructions.

Co-culture experiments with HLE

Sheets of HLE obtained from dispase-treated tissue were dissociated by treatment with 0.25% trypsin in 0.2 g/L EDTA (Invitrogen, VIC, Australia) for 5 min then washed and resuspended in epithelial growth medium (EGF) consisting of a mixture of 3 parts DMEM with 1 part F12 medium (both from Invitrogen, VIC, Australia), supplemented with 10% FBS, 1% penicillin/streptomycin solution, 10 ng/mL EGF, 1% v/v non-essential amino acids, 400 µM L-glutamine (all Invitrogen, VIC, Australia), 6.8 mg 3,3,5-triiodo-L-thyronine sodium salt,

180 µM adenine, 5 µg/mL transferrin, 0.4 µg/mL hydrocortisone, 1 µg/mL insulin (all Sigma-Aldrich, NSW, Australia), and 10^{-5} M isoproterenol (Calbiochem, Merck, VIC, Australia).

The ability of HLS cells to serve as feeder cells for HLE growth was examined in comparison with the gold-standard (murine 3T3 fibroblasts). The murine 3T3 fibroblast feeder cells (ATCC; CCL-92) were maintained in DMEM supplemented with 10% FBS and 400 µM L-glutamine prior to use. HLE cell cultures were established by seeding freshly isolated cells into 75 cm^2 flasks at a density of 5×10^5 cells in the presence of 2×10^6 feeder cells (either 3T3 fibroblasts or HLS cells grown in either 10% FBS medium or MesenCult-XF®). All feeder cells were growth-arrested prior to seeding by gamma irradiation (4 x 25 gray). HLE cultures were grown to approximately 80-90% confluency, before being subcultured for 5 days in the absence of feeder cells and examined by immunocytochemistry for CK3 and ΔNp63 (as described above).

The effects of each feeder cell type on the colony-forming efficiency (CFE) of HLE, was also examined using standard techniques. In brief, standard cultures of HLE were established as above with 3T3 feeders, then seeded at a density of 170 cells per cm^2 into a 6 cm petri dish in the presence of 8.3×10^4 cells/cm^2 of either 3T3 or HLS feeder cells. After 14 days the cultures were fixed in formalin and stained with Rhodamine B Solution (Sigma Aldrich, NSW, Australia) to demonstrate epithelial colonies. The number of colonies (defined as 20 cells or more) was expressed as a percentage of the original seeding density: (number of colonies counted/number of cells plated) × 100.

Results

Establishment of cultures from limbal stroma

Freshly isolated HLS cells cultured in 10% FBS medium adopted a regular spindle-shaped morphology and reached 80-90% confluence within 14 days on average (Fig. 1A). MesenCult-XF® supported similar growth to confluency, but the cells displayed a thinner and more elongated spindle shape than those grown in the presence of serum (Fig. 1D). DK-SFM supported attachment and growth of spindle-shaped cells, but confluency was rarely observed except in a few cultures after approximately 30-50 days (Fig. 1B). Cells grown in serum-free medium supplemented with defined growth factors (abbreviated as 2% B-27 medium), formed non-adherent spherical aggregates, but resulted in poor yields (Fig. 1C). Average yields from at least 3 individual donors at passage (p) 0 in a 24 well plate were 7.6×10^4 for 10% FBS medium, 7.0×10^3 for DK-SFM, 6.0×10^3 for 2% B-27, and 3.4×10^5 for MesenCult-XF®.

Propagation of cultures from limbal stroma

A direct comparison of serial growth when cells were maintained in each of the primary media used for three passages (Fig. 2), revealed highest yields in MesenCult-XF® medium (2.94×10^6 per T75 flask) followed closely by serum-supplemented medium (1.92×10^6 per T75 flask). In contrast, p0 cultures achieving confluency in DK-SFM, subsequently failed to thrive when passaged in this commercial serum-free medium.

Cultures established in the presence of 10% FBS, failed to thrive following transfer to DK-SFM, 2% B-27 medium, or 10 ng/mL FGF-2 (Fig. 3B, 3C, and 3D), but grew well in MesenCult-XF® medium (Fig. 3E). Average cell yields from at least 3 individual donors at p1 in a T25 flask were 1.1×10^6 for 10% FBS, 1.6×10^5 cells for DK-SFM, 9.7×10^3 cells

for 2% B-27 medium, 7.7×10^3 cells for 10 ng/mL FGF-2 medium, and 2.6×10^6 cells for MesenCult-XF®. Cultures that failed to thrive in serum-free medium at p0 also did not improve when transferred to low-serum (1% FBS) containing medium at p1 (data not shown).

Phenotype of cultures from limbal stroma

HLS cells established in 10% FBS medium displayed positive expression of CD90, trace expression of CD141 and CD271, and 81.04% (± 1.65 SEM) of live cells expressed the characteristic MSC markers of $CD34^-$, $CD45^-$, $CD90^+$, $CD73^+$, $CD105^+$ (Fig. 1E). Serum cultivated BM-MSC, as a positive control, were 99% $CD34^-$, $CD45^-$, $CD90^+$, $CD73^+$, $CD105^+$ but negative for CD141 at passage 2 (data not shown). Approximately 25% of HLS cells grown in the presence of serum stained positively for α-sma at passage 2 (Fig. 4A, 4B, and 4C), similarly to BM-MSC (Fig. 4G, 4H, and 4I). HLS cells cultured in DK-SFM also had positive expression of CD90; however, this cell population was 58.43% (± 10.45 SEM) positive for the characteristic MSC markers. DK-SFM cultures contained a quarter of live cells which were positive for CD141 (29.44 ± 8.45%; Fig. 1F). The cultures established in 2% B-27 medium had less CD90 cell surface expression than the previous culture methods; however, they had an increase in CD34 quiescent keratocyte expression, and trace amounts of CD141, CD271, and MSC markers (Fig. 1G). MesenCult-XF® cultures contained 96.04% (± 0.61 SEM) cells which expressed a characteristic MSC phenotype and had positive expression (38.37 ± 0.28%) of CD141 (Fig. 1H). Staining for α-sma was rarely observed in MesenCult-XF® HLS cell cultures at passage 2 (Fig. 4D, 4E, and 4F). Overall, HLS cells cultured in 10% FBS medium and the MesenCult-XF® culture system produced the most consistent phenotype as judged by the relative size of error bars.

For cultures established in serum, marked changes in phenotype were observed when switched to serum-free media. Cultures changed into DK-SFM displayed an increase in

CD34 and CD90 cell surface expression, and a decrease in CD141 and MSC profile expression (Fig. 3G). HLS cell cultures switched to 2% B-27 medium became more uniform with a predominant CD90 phenotype (Fig. 3H). Serum-free cultures supplemented with only FGF-2 contained ~35% cells positive for CD90 (36.91 ± 8.35%) and the MSC markers (32.88 ± 10.45%; Fig. 3I). The 2% B-27 and FGF-2 cultures also had a proportion of cells not positive for any of our specified markers. Cultures switched to MesenCult-XF® contained more cells with the MSC characteristic phenotype and more CD141 expression (Fig. 3J) than cells maintained in serum-supplemented medium. Cells maintained throughout in serum-supplemented medium displayed fewer MSC (71.90 ± 2.10%) at p1 than observed in p0 cultures (>80%), but retained high numbers of CD90 expression (98.24 ± 0.33%; Fig. 3F).

Differentiation potential of cultures from limbal stroma

HLS cells grown in 10% FBS medium and the MesenCult-XF® culture system, along with BM-MSC grown in serum-supplemented medium, displayed expression of osteogenic (Fig. 5B, 5G, and 5L), adipogenic (Fig. 5C, 5H, and 5M), and chondrogenic (Alcian Blue, Fig. 5D, 5I, and 5N; Collagen II, Fig. 5E, 5J, and 5O) potential. Osteogenic and chondrogenic potential was similar among all cohorts; however, 10% FBS medium supported lower adipogenic expression compared with MesenCult-XF® and BM-MSC cultures.

Response of limbal epithelial cells to limbal stromal cells when used as feeders

After thoroughly analysing the phenotype of the limbal stromal cells, we sought to investigate one application of their use, as a feeder layer for HLE cells. Limbal stromal cell cultures grown in 10% FBS medium or MesenCult-XF® medium were able to support the growth of HLE cells comparably to 3T3 feeder cells (Fig. 6D). Based simply upon the number of discrete islands, the 3T3 feeder cells displayed the best CFE with an average of 5.5%

(compared to 0.86% for 10% FBS and 0.48% for MesenCult-XF® feeders); however, a realistic comparison with MesenCult® cultures was not feasible owing to apparent rapid expansion and connectivity of islands. As such the mean diameter of HLE colonies varied greatly between 3T3 feeders (~3mm), 10% FBS feeders (~9mm), and MesenCult-XF® feeders (~1.5cm). Limbal epithelial morphology on all feeder layers remained similar (Fig. 6A, 6B, and 6C).

CK3 was expressed in all HLE cultures, with faint expression on lower layers and brighter expression on stratified portions of the cell layers (Fig. 7). 3T3 feeders contained the most stratified epithelium (Fig. 7A, 7B, and 7C), with the limbal feeders having smaller proportions of CK3 bright cells (Fig. 7E, 7F, 7H, and 7I). ΔNp63 nuclear expression was seen in HLE cells cultured on all feeder layers (Fig. 8). However, the 10% FBS cohort displayed larger and more sparse HLE nuclei (Fig. 8E) compared with 3T3 (Fig. 8B) and MesenCult-XF® (Fig. 8H) feeder layers which produced smaller and more densely packed HLE nuclei.

Discussion

The limbal epithelium has been widely recognized as a source of epithelial progenitor cells for corneal tissue engineering (4,6,7,21,22). Hence, the recent discovery of stromal progenitor cells with characteristics similar to that of BM-MSC is likely to lead to further innovative therapies (8,10). Potential applications of L-MSC include use as feeder cells for *ex vivo* expansion of epithelial progenitor cells, thus replacing the requirement for murine 3T3 feeder cells. Alternatively, L-MSC could be used in their own right as a cellular therapy for reconstruction of the limbal or corneal stroma. An important step in developing these applications is to establish appropriate techniques for L-MSC isolation and culture. Thus we have evaluated in this study a range of current methods for growing HLS cells along with a

recently developed commercial product for growing human-derived MSC. Our results demonstrate that while serum-supplemented growth medium provides an effective method for generating and propagating L-MSC, these cells can be grown in larger quantities and to a higher degree of purity using the MesenCult-XF® culture system. Nevertheless, the unexpected finding of elevated CD141 expression in cultures grown using the MesenCult-XF® system raises some significant questions for further exploration.

To understand the significance of our results, the anatomy of the limbus should be considered. Unlike the central corneal stroma which contains relatively few cell types (including resident $CD34^+$ keratocytes), the limbus contains peripheral keratocytes, fibroblasts, melanocytes, immune cells including dendritic cells, and various cell types associated with blood vessels (vascular endothelial cells, pericytes and smooth muscle cells) and nerve fibers (Schwann cells and fibroblasts). Moreover, the potential for contaminating limbal epithelial cells or differentiated stromal cells must also be considered.

The phenotype of HLS cell cultures grown in the presence of 10% FBS is similar to that for BM-MSC including the expression of α-sma and suggests that few, if any, other cell types are present with the exception of differentiated fibroblasts and myofibroblasts. Based upon the previous results of others (Table 1), we had predicted that use of serum-free growth medium supplemented with a defined combination of growth factors, would increase the number and purity of progenitor-like cells with either an MSC or keratocyte profile, but unfortunately this proved not to be the case. These serum-free techniques proved to be unsuitable for routine cultivation of any HLS cell type due to the poor growth observed. The MesenCult-XF® culture system was developed with the intent of providing a more defined and higher performing alternative to the use of serum-supplemented medium for routine cultivation of human-derived MSC. Interestingly, Pal et al. (23) concluded that MesenCult-XF® performs less well that serum-supplemented growth medium for BM-MSC due to

apparent senescence after approximately 6 passages. Based upon our prior clinical experience of cultured HLE cells, we did not consider the need to grow stromal cells beyond passage 3 in the current study, and thus it remains possible that extended cultivation might reveal similar results. In the absence of such further data, however, we conclude that, based upon the superior growth and increased purity of L-MSC (including lower numbers of myofibroblasts) using the MesenCult-XF® culture system, this should be considered as a alternative to use of serum-supplemented medium. Nevertheless, the unexpected increase in CD141 expression warrants further investigation.

The cell surface marker CD141, or thrombomodulin is a co-factor of thrombin-catalyzed activation of protein C, which in turn inhibits the pro-coagulant functions of thrombin. Thrombomodulin is located on cells such as endothelium, keratinocytes, osteoblasts, and macrophages, in which it may be involved in cell differentiation or in inflammation (24). Based upon this knowledge it is possible that a number of cells grown in MesenCult-XF® may have commenced partial differentiation into vascular endothelial cells. If true, this could be problematic for use of this growth medium in therapies for treating the cornea since the corneal stroma is normally avascular and indeed a vascularised corneal stroma (corneal neovascularisation) is a common feature of chronic corneal disease. Alternatively, the use of this medium may well be appropriate for reconstructing rings of limbal tissue since vascularisation of this tissue is considered a contributor to maintaining the epithelial stem cell niche.

We have previously reported that HLS cells cultured in serum-supplemented media can adequately support the growth of HLE cells (9). In this study, L-MSC supported an HLE layer which retains vital similarities in HLE cell phenotype and morphology compared to those produced on 3T3 feeder cells. Importantly, it is unlikely that the epithelial islands could have arisen in part from mesenchymal-to-epithelial transformation since all feeder cell used

in the study were growth arrested by gamma irradiation prior to use. Furthermore, the clinical efficacy of cultivated HLE grafts relies upon the phenotype of the grafted cells. Importantly, there was evidence of CK3 expression and abundant expression of the progenitor marker ΔNp63, which is highly desirable for clinical efficacy (9,22). These results also concur with those reported in the use of BM-MSC as a feeder layer for HLE (25).

In conclusion, our studies demonstrate the ability to consistently culture L-MSC serum-free using the MesenCult-XF® culture system while maintaining the defined MSC characteristic phenotype; however, further investigation of CD141 expression is warranted. L-MSC also supported the cultivation of limbal epithelium of a clinically appropriate phenotype. Optimisation of this method could be achieved by determining a serum-free method which supports the co-culture of L-MSC and HLE cells. The potential benefits of L-MSC in tissue engineered corneal models should also be explored.

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Declaration of Interest

The authors report no conflicts of interest.

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Table 4.1. Summary of methods used previously to grow stromal cells from the cornea and corneal limbus. Abbreviations are as follows: BrDU – bromodeoxyuridine; DMEM – Dulbecco's Modified Eagle Medium; FBS – foetal bovine serum; EGF – epidermal growth factor; PDGF – platelet-derived growth factor; ITS – insulin, transferrin, selenium; LIF – leukaemia inhibitory factor; BSA – bovine serum albumin; FGF-2 – fibroblast growth factor 2; HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Reference	Cell Source	Isolation method	Culture method	Propagation	Phenotype/ Multi-potency
Choong *et al.* (2007)	Human corneal stroma	Corneal explant technique. Corneal stromal was chopped into fine pieces.	DMEM supplemented with 10% FBS, 0.05 U/mL penicillin and 0.05 U/mL streptomycin.	Passaged 4 times.	$CD13^+$, $CD29^+$, $CD44^+$, $CD56^+$, $CD73^+$, $CD90^+$, $CD105^+$, $CD133^+$. $HLA\text{-}DR^-$, $CD34^-$, $CD117^-$ & $CD45^-$. Multi-potent.
Polisetty *et al.* (2008)	Human limbal biopsy	Established from explant then enriched by passaging.	Human corneal epithelial medium with 10% FBS.	Passaged 6 times.	$CD105^+$, $CD106^+$, $CD54^+$, $CD166^+$, $CD90^+$, $CD29^+$, $CD71^+$, $pax6^+$. $p75^-$, $SSEA1^-$, $Tra\text{-}1\text{-}61^-$, $Tra\text{-}1\text{-}81^-$, $CD31^-$, $CD34^-$, $CD45^-$, $CD11a^-$,

					CD11c$^-$, CD14$^-$, CD138$^-$, Flk1$^-$, Flt1$^-$, VE-Cadherin$^-$. Multi-potent.
Ainscough *et al.* (2011)	Human limbal stroma	2 mm stromal biopsies from the limbus were digested for 48 h in 0.05% collagenase prepared in growth medium.	DMEM supplemented with 10% FBS.	Passaged 16 times.	Vimentin$^+$, CD90$^+$. CD34$^-$, CD45$^-$, CD141$^-$, CD271$^-$.
Du *et al.* (2005)	Human corneal stroma	Minced into 2 mm cubes. Stroma was digested up to 3 h at 37ºC in DMEM with 1 mg/mL collagenase + 0.2 mg/mL testicular hyaluronidase.	DMEM/MCDB-201 supplemented with 2% FBS, 10 ng/mL EGF, 10 ng/mL PDGF, 5 ug/mL ITS, 1,000 U/mL LIF, x1 linoleic acid-BSA, 0.1 mM ascorbic acid-2-phosphate, 10-8 M dexamethansone, with antibiotics. ABCG2$^+$ subpopulation subcultivated in Advanced DMEM supplemented with 10 ng/mL FGF-2.	Unknown.	keratocan$^+$; keratan sulfate$^+$; ALDH$^+$. Multi-potent.

Kawakita *et al.* (2006)	Primate corneal stroma	Digested at 37°C for 16 h in 2.5 mL of DMEM containing 1 mg/mL collagenase A, 20 mM HEPES, 50 g/mL gentamicin, and 1.25 g/mL amphotericin B.	Defined Keratinocyte Serum-Free Medium.	Passaged 14 times.	$CD34^{+}$, $keratocan^{+}$, $ALDH^{+}$
Mimura *et al.* (2008)	Rabbit corneal stroma	Overnight digestion in 0.02% collagenase.	DMEM/F12 supplemented with B-27, 20 ng/ml EGF, 40 ng/mL FGF and 0.8% methylcellulose gel matrix.	Unknown.	$BrDU^{+}$, $keratocan^{+}$, $vimentin^{+}$.
Funderburgh *et al.* (2008)	Bovine corneal stroma	Digestion for 1 hour in 1 mg/mL collagenase. Placed overnight on ice, before 2-4 h incubation with 10 mg/mL collagenase	Primary keratocytes were suspended in protein free DMEM plus antibiotics, or in advanced DMEM (with ITS) plus antibiotics. Cells were placed in tissue culture dishes coated with polyHEMA at 37°C.	Unknown.	$BrDU^{+}$, $keratocan^{+}$, keratan $sulfate^{+}$, $lumican^{+}$.

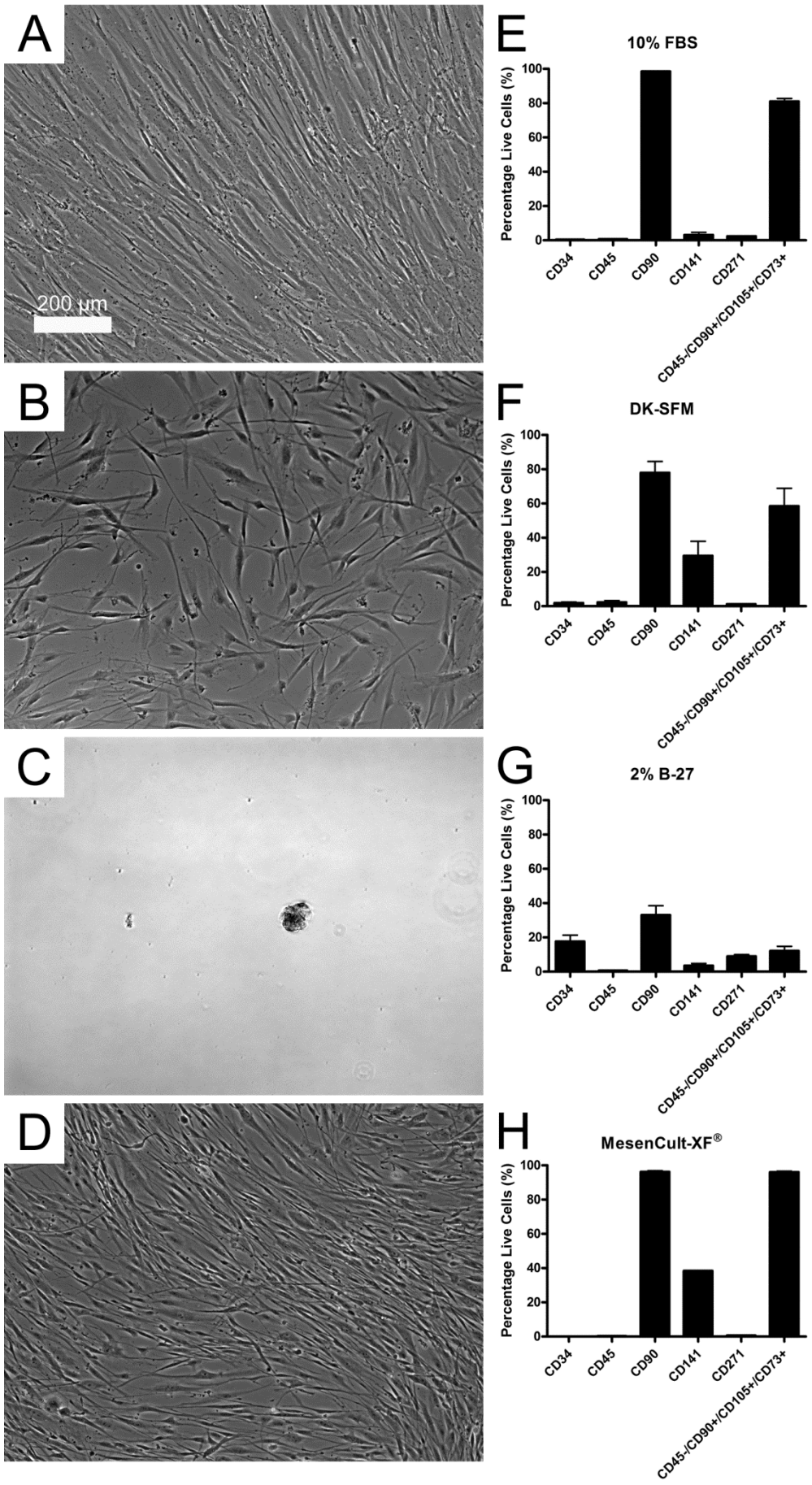
A
200 µm
B
C
D
E
10% FBS
Percentage Live Cells (%)
100
80
60
40
20
0
CD34
CD45
CD90
CD141
CD271
CD45-/CD90+/CD105+/CD73+
F
DK-SFM
G
2% B-27
H
MesenCult-XF®

Figure 1. Morphology and phenotype of cultures established from limbal stroma at passage 0. Representative phase contrast images of cultures established in either DMEM/F12 with 10% foetal bovine serum (10% FBS medium; part A), Defined Keratinocyte Serum-Free Medium (DK-SFM; part B), DMEM/F12 medium containing B-27 supplement, insulin, transferrin, selenium (ITS), epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF-2) (2% B-27 medium; parts C & G), or using the MesenCult-XF® serum-free culture system designed for human derived mesenchymal stromal cells (MSC; part D). (Parts E, F, G & H) Corresponding percentage of live cells in cultures that are positive for CD34, CD45, CD90, CD141, CD271, or the MSC characteristic markers ($CD45^{-}CD73^{+}CD90^{+}CD105^{+}$) by flow cytometry. Bars are mean ± SEM. All flow cytometry was performed on a minimum of three individual donors with three replicates per donor. Scale bar = 200 µm.

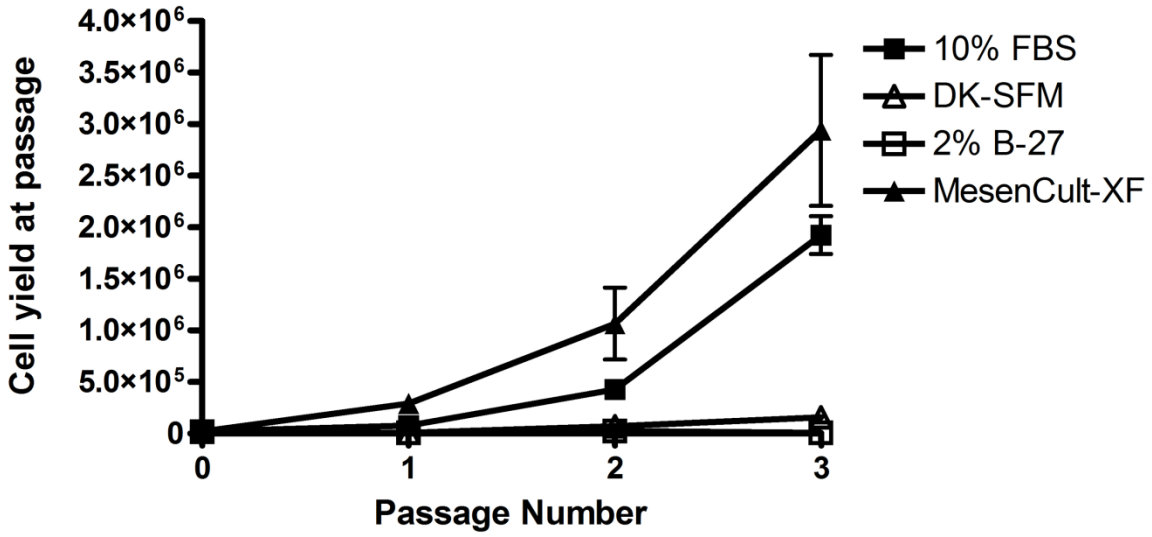

Figure 2. Response of limbal stromal cell cultures to serial propagation. Cultures were propagated in either DMEM/F12 with 10% foetal bovine serum (10% FBS medium) (■), Defined Keratinocyte Serum-Free Medium (DK-SFM; Δ), DMEM/F12 medium supplemented with 2% B-27, insulin, transferrin, selenium (ITS), epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF-2) (2% B-27 medium; □), or MesenCult-XF® medium (▲). Values at each passage point indicate either the total number of cells initially seeded into culture (p0), or total number of cells subsequently harvested after sequential growth in one well of a 24-well culture plate (p1), a 25 cm^2 culture flask, or a 75 cm^2 culture flask. All cultures were harvested together when confluency was achieved in serum-supplemented medium. Bars represent mean ± SEM for up to 8 different tissue donors.

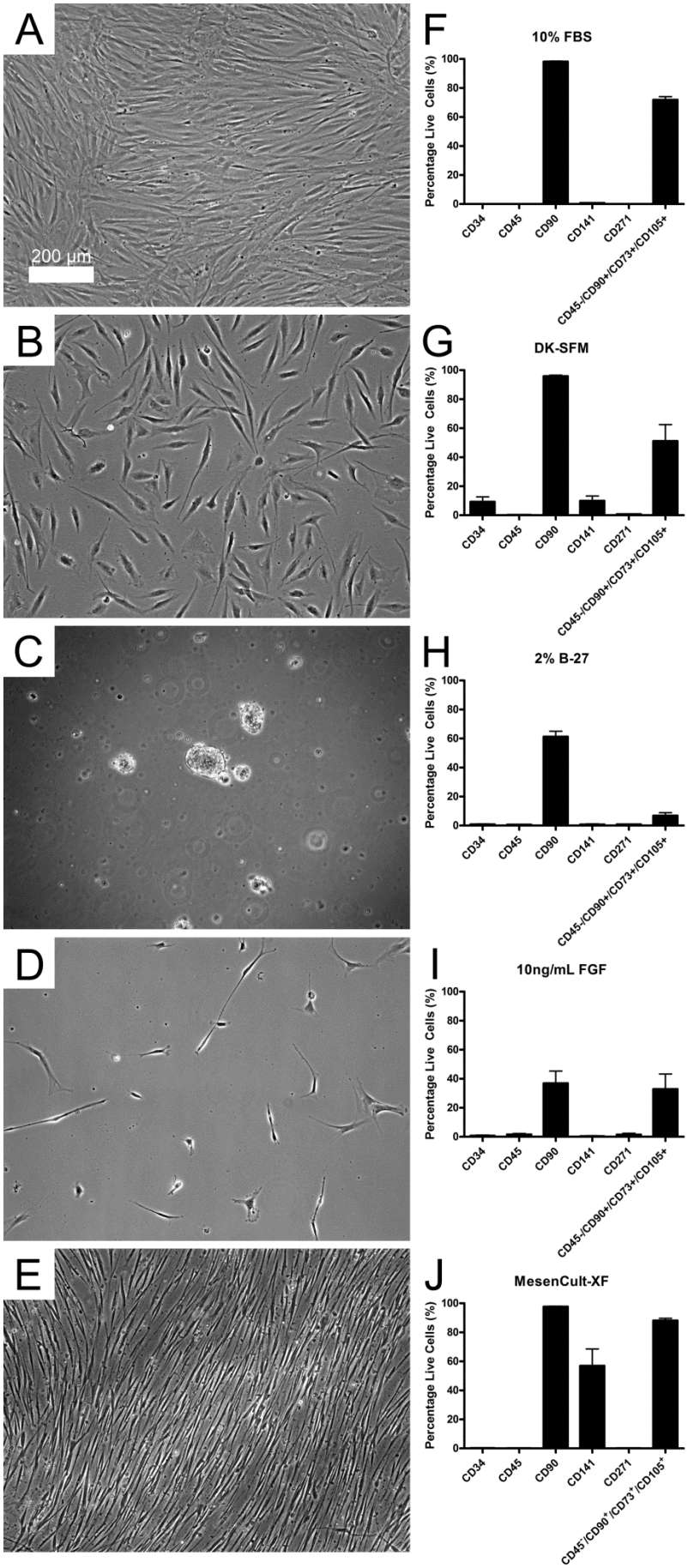
A
200 µm
B
C
D
E
F
10% FBS
G
DK-SFM
H
2% B-27
I
10ng/mL FGF
J
MesenCult-XF
Percentage Live Cells (%)
0
20
40
60
80
100
CD34
CD45
CD90
CD141
CD271
CD45-/CD90+/CD73+/CD105+
CD45-/CD90+/CD73+/CD105+

Figure 3. Morphology and phenotype of cultures established from limbal stroma in serum-supplemented medium before propagation in serum-free medium. Cultures were established in serum-supplemented growth medium, before being passaged into either DMEM/F12 with 10% foetal bovine serum (10% FBS medium, part A), Defined Keratinocyte Serum-Free Medium (DK-SFM; part B), DMEM/F12 medium supplemented with 2% B-27, insulin, transferrin, selenium (ITS), epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF-2) (2% B-27 medium; parts C & H), DMEM/F12 supplemented with 10 ng/mL FGF-2 (part D), or using the MesenCult-XF® serum-free culture system (part E). (Parts F, G, H, I & J) Corresponding percentage of live cells in cultures that are positive for CD34, CD45, CD90, CD141, CD271, or the characteristic mesenchymal stromal cell markers ($CD45^-$ $CD73^+CD90^+CD105^+$) by flow cytometry. Bars are mean ± SEM. All flow cytometry was performed on a minimum of three individual donors with three replicates per donor. Scale bar = 200 µm.

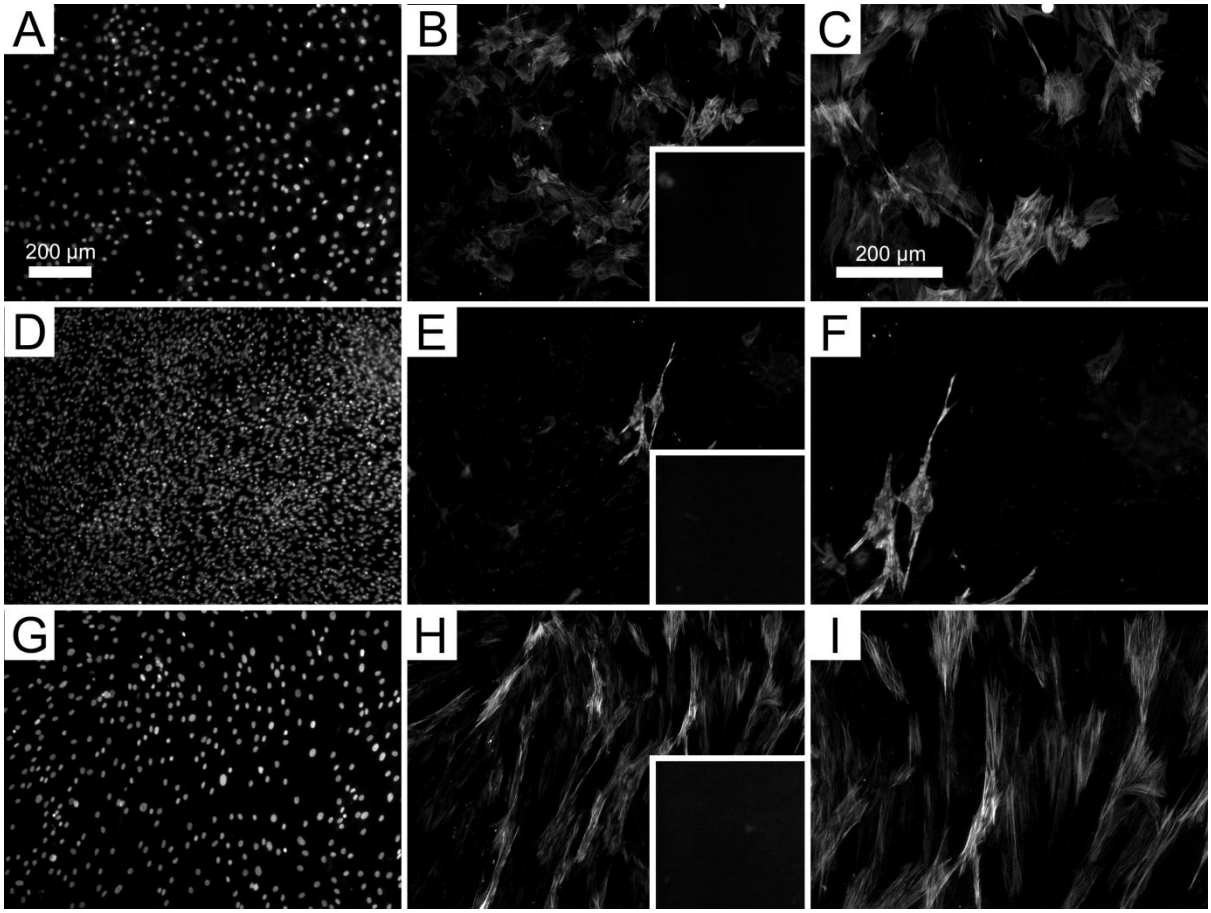

Figure 4. Expression of α-sma in limbal stromal cultures. Demonstration of Hoechst nuclear staining (parts A, D & G) and alpha smooth muscle actin (α-sma; parts B, E & H) expression by immunocytochemistry in passage 2 cultures of limbal mesenchymal stromal cells (MSC) grown in either serum-supplemented growth medium (parts A & B) or using the MesenCult-XF® culture system (parts D & E), compared with passage 2 bone marrow derived MSC (parts G & H). Inserts in parts B, E & H display negative controls for respective cell types. Parts C, F & I show a magnified image of that in parts B, E & H. Scale bars = 200 µm. Scale bar in part C corresponds to magnified images in parts C, F & I.

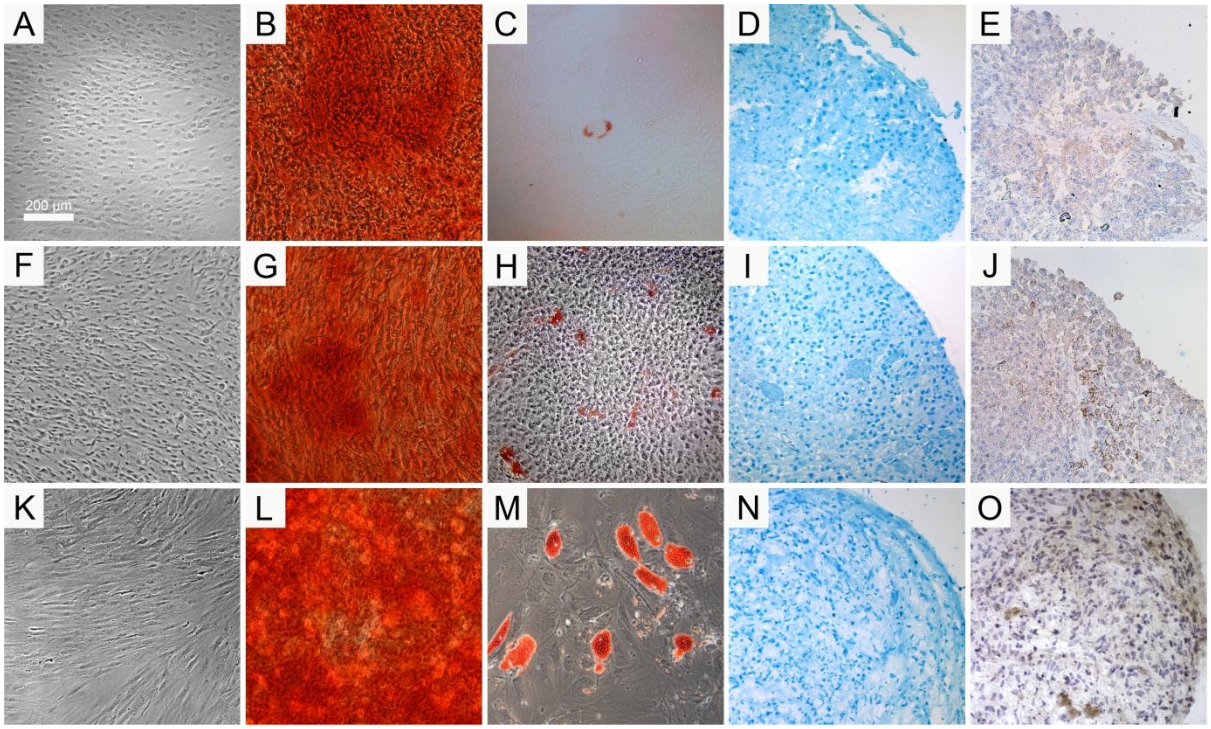

Figure 5. Demonstration of multi-potency by stromal cell cultures derived from limbal tissue**.** Limbal mesenchymal stromal cell (MSC) cultures established in either serum-supplemented growth medium (parts A to E), or using the MesenCult-XF® culture system (parts F to J) were grown for 3 weeks under either osteogenic (parts B, G & L), adipogenic (parts C, H & M) or chondrogenic conditions (Alcian Blue, parts D, I & N; Collagen II, parts E, J & O) before staining with Alizarin Red (areas of calcification), Oil-Red-O (lipid droplets), Alcian Blue (acid mucosubstances), or Collagen Type II (marker of cartilage extracellular matrix) respectively. Corresponding results using BM-MSC are shown in parts K to O. Scale bar = 200 µm.

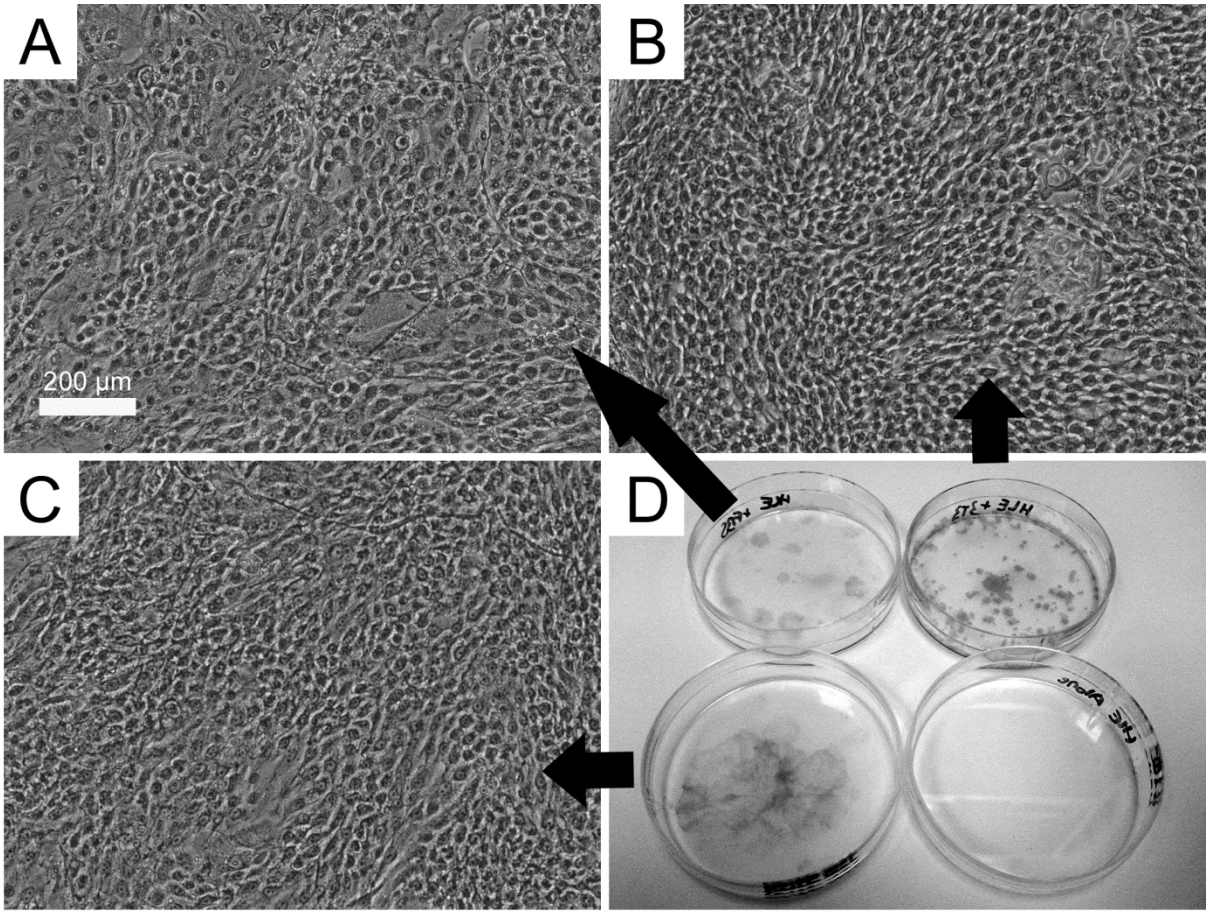

Figure 6. Human limbal epithelial (HLE) colony formations supported by limbal stromal feeders. Results of attempt to perform colony forming efficiency (CFE) assays with HLE cells co-cultured with either stromal cell cultures established in presence of serum (part A), murine 3T3 fibroblasts (part B), or stromal cell cultures established using the MesenCult-XF® culture system (part C). Corresponding images of each culture dish including example of HLE seeded alone is displayed in part D. The confluency of HLE achieved in the presence of MesenCult-XF-derived stromal cells prevented an accurate comparison of colony number with other conditions. Scale bar = 200 µm.

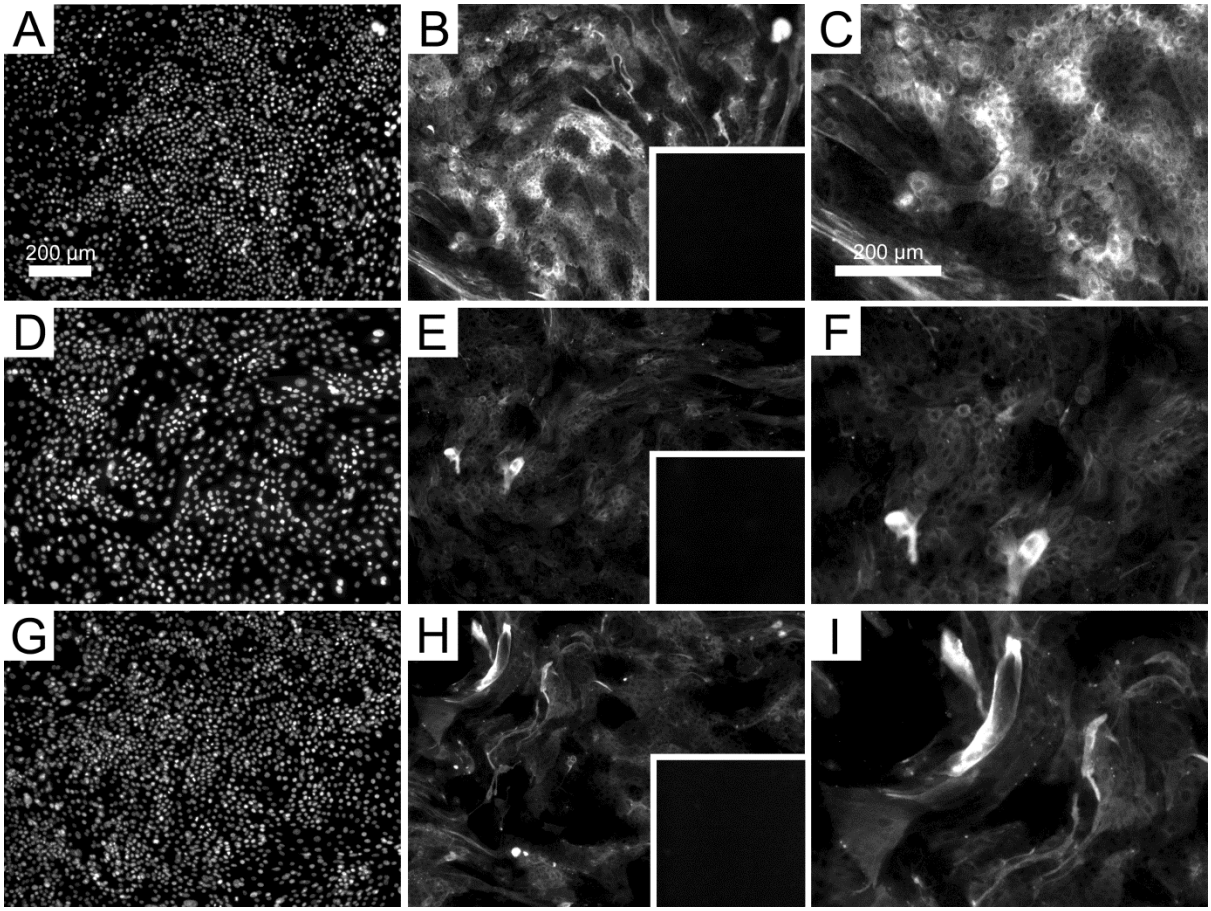

Figure 7. Expression of cytokeratin 3 in limbal stromal cultures. Confirmation of corneal phenotype (via immunostaining for cytokeratin 3) in human limbal epithelial cultures after growth in the presence of either murine 3T3 fibroblasts (part B), limbal stromal cells grown with 10% foetal bovine serum (part E), or limbal stromal cells grown using the MesenCult-XF® system (part H). Corresponding staining with Hoechst nuclear dye is displayed in parts A, D and G respectively. Inserts in parts B, E & H display negative controls for respective cell types. Parts C, F & I show a magnified image of that in parts B, E & H. Scale bars = 200 µm. Scale bar in part C corresponds to magnified images in parts C, F & I.

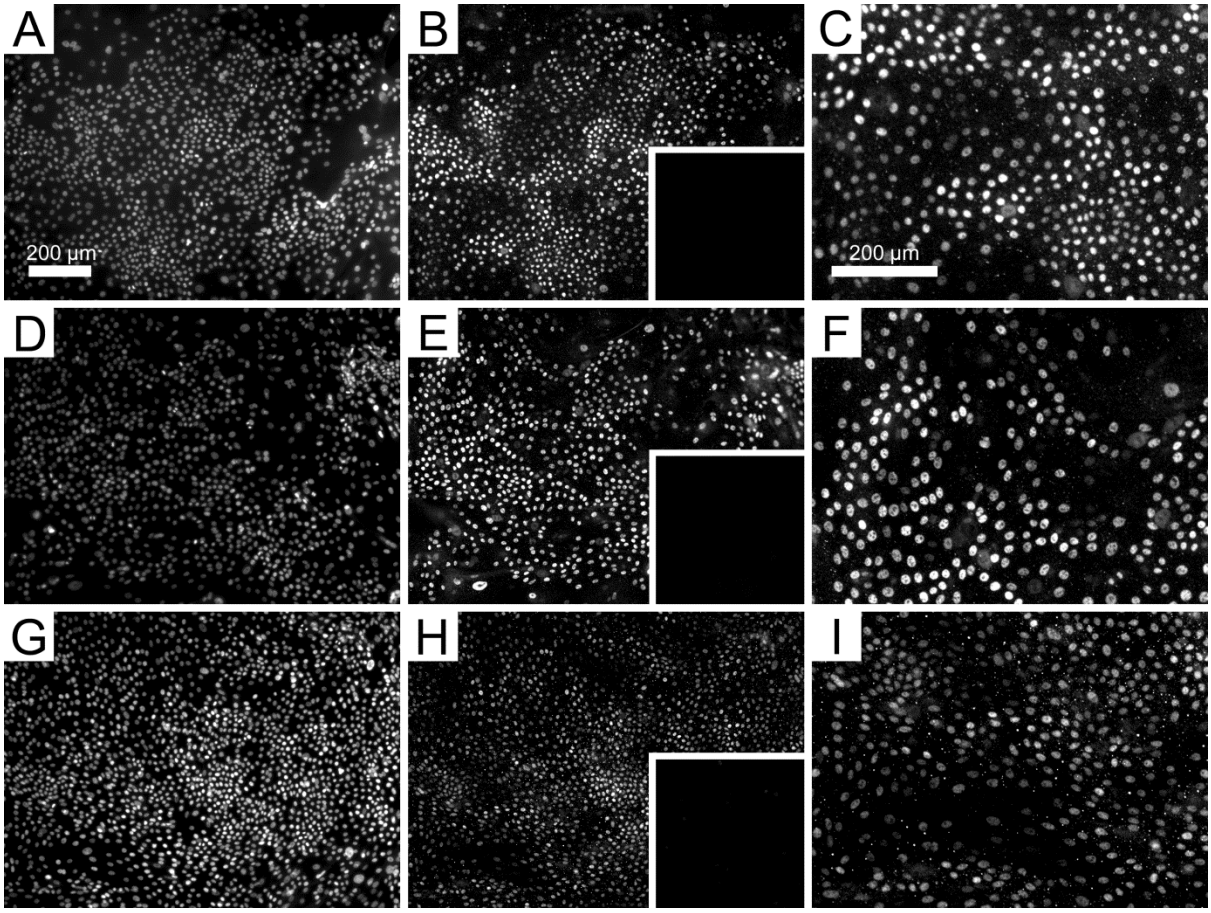

Figure 8. Expression of ΔNp63 in limbal stromal cultures. Examination of progenitor cell numbers (via immunostaining for ΔNp63) in human limbal epithelial cultures after growth in the presence of either murine 3T3 fibroblasts (part B), limbal stromal cells grown with 10% foetal bovine serum (part E), or limbal stromal cells grown using the MesenCult-XF® system (part H). Corresponding staining with Hoechst nuclear dye is displayed in parts A, D and G respectively. Inserts in parts B, E & H display negative controls for respective cell types. Parts C, F & I show a magnified image of that in parts B, E & H. Scale bars = 200 µm. Scale bar in part C corresponds to magnified images in parts C, F & I.